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PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>8.67993/002</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/GB 99/ 01228</b>	International filing date (day/month/year) <b>22/04/1999</b>	(Earliest) Priority Date (day/month/year) <b>22/04/1998</b>
Applicant <b>MARSDEN, John, Christopher et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

contained in the international application in written form.

filed together with the international application in computer readable form.

furnished subsequently to this Authority in written form.

furnished subsequently to this Authority in computer readable form.

the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2.  Certain claims were found unsearchable (See Box I).

3.  Unity of invention is lacking (see Box II).

4. With regard to the title,

the text is approved as submitted by the applicant.

the text has been established by this Authority to read as follows:

**ULTRASOUND CONTRAST AGENT DISPERSIONS COMPRISING GAS AND DESTABILISING AGENT**

5. With regard to the abstract,

the text is approved as submitted by the applicant.

the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

as suggested by the applicant.

because the applicant failed to suggest a figure.

because this figure better characterizes the invention.

None of the figures.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01228

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 98 17324 A (MARDSEN JOHN CHRISTOPHER ; ERIKSEN MORTEN (NO); OESTENSEN JONNY (NO) 30 April 1998 (1998-04-30) cited in the application page 4, line 5 - page 17, line 2 page 29, line 6 - page 34, line 35; claims 1-29 ---	1-22
X	EP 0 212 568 A (MAX PLANCK GESELLSCHAFT) 4 March 1987 (1987-03-04) the whole document ---	1-12
X	EP 0 365 467 A (SCHERING AG) 25 April 1990 (1990-04-25) page 4, line 39 - page 5, line 19; claims 1,6 ---	1-12
		-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## ° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

23 August 1999

13/09/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
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Authorized officer

Engl, B

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01228

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 07072 A (SCHERING AG ;WEITSCHIES WERNER (DE); HEIDMANN DIETER (DE); HAUFF P) 16 March 1995 (1995-03-16) page 1, line 33 – page 2, line 12 page 3, line 31 – page 4, line 17 ---	1-22
Y	WO 98 10799 A (IMARX PHARMACEUTICAL CORP) 19 March 1998 (1998-03-19) page 6, line 30 – page 7, line 30 page 71, line 9-24 page 78, line 24-30 ---	13-22
Y	US 5 685 310 A (PORTER THOMAS R) 11 November 1997 (1997-11-11) column 2, line 30-39 column 4, line 16-28 ---	1-17
Y	WEI K ET AL: "Quantification of Myocardial Blood Flow With Ultrasound-Induced Destruction of Microbubbles Administered as a Constant Venous Infusion" CIRCULATION, vol. 5, no. 97, 10 February 1998 (1998-02-10), pages 473-483, XP002077428 ISSN: 0009-7322 the whole document ---	1-17
Y	WEY K ET AL: "Use of Microbubble Destruction as a Novel Approach for Quantification of Myocardial Perfusion With Contrast Echocardiography During Venous Infusion of Contrast" JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, 1 February 1997 (1997-02-01), XP002077429 ISSN: 0735-1097 the whole document ---	1-17
A	VANDEBERG B. ET AL.: "Myocardial risk area and peak gray level measurement by contrast echocardiography: Effect of microbubble size and concentration, injection rate, and coronary vasodilation" AMERICAN HEART JOURNAL, vol. 115, 1988, page 733-739 XP002112966 the whole document -----	1-22

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

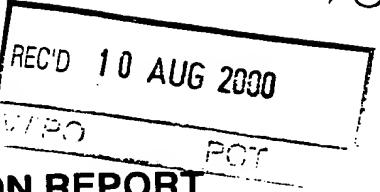
International Application No

PCT/GB 99/01228

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9817324	A 30-04-1998	AU 4714797 A NO 991869 A		15-05-1998 02-06-1999
EP 0212568	A 04-03-1987	DE 3529195 A AT 69169 T DE 3682342 A JP 62155224 A US 4832941 A		26-02-1987 15-11-1991 12-12-1991 10-07-1987 23-05-1989
EP 0365467	A 25-04-1990	DE 3834705 A AT 93145 T AU 627456 B AU 3390089 A CA 1338507 A CN 1044225 A,B DK 99789 A ES 2059814 T FI 890749 A IE 65243 B JP 2005630 C JP 2145528 A JP 7037394 B KR 9701433 B LU 90004 A NO 178139 B PT 91523 A,B US 5141738 A		12-04-1990 15-09-1993 27-08-1992 12-04-1990 06-08-1996 01-08-1990 08-04-1990 16-11-1994 08-04-1990 04-10-1995 11-01-1996 05-06-1990 26-04-1995 06-02-1997 04-03-1997 23-10-1995 30-04-1990 25-08-1992
WO 9507072	A 16-03-1995	DE 4330958 A DE 4416818 A AU 7655194 A AU 7729998 A CA 2171303 A EP 0717617 A HU 74509 A JP 9502191 T NO 960973 A		16-03-1995 16-11-1995 27-03-1995 10-09-1998 16-03-1995 26-06-1996 28-01-1997 04-03-1997 08-03-1996
WO 9810799	A 19-03-1998	AU 4089897 A AU 4235697 A WO 9810798 A US 5846517 A		02-04-1998 02-04-1998 19-03-1998 08-12-1998
US 5685310	A 11-11-1997	US 5560364 A AU 5383296 A EP 0868200 A WO 9635457 A US 5740807 A		01-10-1996 29-11-1996 07-10-1998 14-11-1996 21-04-1998

## PATENT COOPERATION TREATY

PCT



## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference  8.67993/002	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No.  PCT/GB99/01228	International filing date (day/month/year)  22/04/1999	Priority date (day/month/year)  22/04/1998
International Patent Classification (IPC) or national classification and IPC  A61K49/00		
Applicant  NYCOMED IMAGING AS & AL.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

- I  Basis of the report
- II  Priority
- III  Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV  Lack of unity of invention
- V  Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI  Certain documents cited
- VII  Certain defects in the international application
- VIII  Certain observations on the international application

Date of submission of the demand  12/11/1999	Date of completion of this report  08.08.2000
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Engl, B  Telephone No. +49 89 2399 8283



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB99/01228

**I. Basis of the report**

1. This report has been drawn on the basis of (substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

**Description, pages:**

1-24 as originally filed

**Claims, No.:**

7-22 as originally filed

1-6 as received on 19/05/2000 with letter of 17/05/2000

2. The amendments have resulted in the cancellation of:

the description, pages:  
 the claims, Nos.:  
 the drawings, sheets:

3.  This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims 1-22
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-22
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-22
	No:	Claims

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB99/01228

2. Citations and explanations

**see separate sheet**

**Concerning Section I/4:**

There are no unified criteria under the PCT for assessing the industrial applicability of claims directed to methods of diagnosis (claims 17 to 20) or methods of therapeutic treatment of the human or animal body (claims 21, 22).

**Concerning Section V:**

Combinations of injectable aqueous gas dispersions and separately administrable destabilising substances are neither taught nor suggested in the available prior art. The present claimed subject-matter is therefore considered to be novel and inventive (Article 33 (2) and (3) PCT).

Subject to what has been stated in Section I/4 above, the claimed subject-matter appears to be industrially applicable (Article 33 (4) PCT) since it can be worked in the pharmaceutical industry.

Claims:

1. A combined presentation for simultaneous, separate or sequential use as an ultrasound contrast agent, said preparation comprising:

5           i) an injectable aqueous gas dispersion; and  
          ii) an administrable substance or substances capable of destabilising said dispersed gas so as at least transiently to increase the size thereof.

10

2. A combined preparation as claimed in claim 1 wherein the dispersed gas comprises air, nitrogen, oxygen, carbon dioxide, hydrogen, an inert gas, a sulphur fluoride, selenium hexafluoride, an optionally halogenated silane, an optionally halogenated low molecular weight hydrocarbon, a ketone, an ester or a mixture of any of the foregoing.

15

20 3. A combined preparation as claimed in claim 2 wherein the dispersed gas comprises sulphur hexafluoride or a perfluorocarbon.

25

4. A combined preparation as claimed in claim 3 wherein said perfluorocarbon is perfluoropropane, perfluorobutane or perfluoropentane.

30

5. A combined preparation as claimed in any of the preceding claims wherein the dispersed gas is stabilised by an initially coalescence-resisting surface membrane, a filmogenic protein, a polymer material, a non-polymeric and non-polymerisable wall-forming material or a surfactant.

35 6. A combined preparation as claimed in claim 5 wherein said surfactant comprises at least one phospholipid.

*Replies by AT34*

## ENT COOPERATION TRE,

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**NOTIFICATION OF ELECTION**

## From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 18 January 2000 (18.01.00)	in its capacity as elected Office
<b>International application No.</b> PCT/GB99/01228	<b>Applicant's or agent's file reference</b> 8.67993/002
<b>International filing date (day/month/year)</b> 22 April 1999 (22.04.99)	<b>Priority date (day/month/year)</b> 22 April 1998 (22.04.98)
<b>Applicant</b>	
SKURTVEIT, Roald et al	

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:

12 November 1999 (12.11.99)

in a notice effecting later election filed with the International Bureau on:

2. The election  was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32 2(b)

<p><b>The International Bureau of WIPO</b>  <b>34, chemin des Colombettes</b>  <b>1211 Geneva 20, Switzerland</b></p> <p>Facsimile No.: (41-22) 740.14.35</p>	<p><b>Authorized officer</b></p> <p><b>S. Mafla</b></p> <p>Telephone No.: (41-22) 338.83.38</p>
---	---

## F. FENT COOPERATION TREA.

PCT

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)Date of mailing (day/month/year)  
15 June 2000 (15.06.00)

From the INTERNATIONAL BUREAU

To:

MARDEN, John, Christopher  
Frank B. Dehn & Co.  
179 Queen Victoria Street  
London EC4V 4EL  
ROYAUME-UNIApplicant's or agent's file reference  
8.67993/002

## IMPORTANT NOTIFICATION

International application No.  
PCT/GB99/01228International filing date (day/month/year)  
22 April 1999 (22.04.99)

## 1. The following indications appeared on record concerning:

the applicant  the inventor  the agent  the common representative

Name and Address  
MARDEN, John, Christopher  
Frank B Dehn & Co.  
179 Queen Victoria Street  
London EC4V 4EL  
United KingdomState of Nationality  
GBState of Residence  
GB

Telephone No.

Facsimile No.

Teleprinter No.

## 2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

the person  the name  the address  the nationality  the residence

Name and Address

State of Nationality

State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

## 3. Further observations, if necessary:

Deleted from the records as applicant/inventor.

## 4. A copy of this notification has been sent to:

<input checked="" type="checkbox"/> the receiving Office	<input type="checkbox"/> the designated Offices concerned
<input type="checkbox"/> the International Searching Authority	<input checked="" type="checkbox"/> the elected Offices concerned
<input checked="" type="checkbox"/> the International Preliminary Examining Authority	<input type="checkbox"/> other:

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer I. Britel
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> :  A61K 49/00		A1	(11) International Publication Number: <b>WO 99/53964</b>  (43) International Publication Date: 28 October 1999 (28.10.99)
<p>(21) International Application Number: PCT/GB99/01228</p> <p>(22) International Filing Date: 22 April 1999 (22.04.99)</p> <p>(30) Priority Data: 9808582.2 22 April 1998 (22.04.98) GB</p> <p>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/084,881 (CIP) Filed on 8 May 1998 (08.05.98)</p> <p>(71) Applicant (for GB only): MARSDEN, John, Christopher [GB/GB]; Frank B. Dehn &amp; Co., 179 Queen Victoria Street, London EC4V 4EL (GB).</p> <p>(71) Applicant (for all designated States except US): NYCOMED IMAGING AS [NO/NO]; Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): SKURTVEIT, Roald [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). HJELSTUEN, Olaug [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box</p>		<p>4220 Torshov, N-0401 Oslo (NO). ØSTENSEN, Jonny [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO).</p> <p>(74) Agents: MARSDEN, John, Christopher et al.; Frank B. Dehn &amp; Co., 179 Queen Victoria Street, London EC4V 4EL (GB).</p> <p>(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	
<p>(54) Title: ULTRASOUND CONTRAST AGENT DISPERSIONS COMPRISING GAS AND DESTABILISING AGENT</p> <p>(57) Abstract</p> <p>Ultrasonic visualisation of a subject, particularly of perfusion in the myocardium and other tissues, is performed using novel gas-containing contrast agent preparations which provide controllable and temporary growth of the gas phase <i>in vitro</i> following administration and can therefore act as deposited perfusion tracers. The preparations comprise an injectable aqueous gas dispersion and an administrable substance or substances capable of destabilising the dispersed gas to provide temporary growth thereof. In cardiac perfusion imaging the preparations may advantageously be coadministered with vasodilator drugs such as adenosine in order to enhance the differences between return signal intensity from normal and hypoperfused myocardial tissue respectively.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
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CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## ULTRASOUND CONTRAST AGENT DISPERSIONS COMPRISING GAS AND DESTABILISING AGENT

5 This invention relates to ultrasound imaging, more particularly to novel contrast agent preparations and their use in ultrasound imaging, for example in visualising tissue perfusion.

10 It is well known that contrast agents comprising dispersions of microbubbles of gases are particularly efficient backscatterers of ultrasound by virtue of the low density and ease of compressibility of the microbubbles. Such microbubble dispersions, if 15 appropriately stabilised, may permit highly effective ultrasound visualisation of, for example, the vascular system and tissue microvasculature, often at advantageously low doses.

20 The use of ultrasonography to measure blood perfusion (i.e. blood flow per unit of tissue mass) is of potential value in, for example, tumour detection, tumour tissue typically having different vascularity from healthy tissue, and studies of the myocardium, e.g. to detect myocardial infarctions. A problem with the 25 application of existing ultrasound contrast agents to cardiac perfusion studies is that the information content of images obtained is degraded by attenuation caused by contrast agent present in the ventricles of the heart.

30 In our copending International Patent Publication No. WO-A-9817324, the contents of which are incorporated herein by reference, we have disclosed that ultrasonic visualisation of a subject, in particular of perfusion in the myocardium and other tissues, may be achieved and/or enhanced by means of gas-containing contrast 35 agent preparations which promote controllable and temporary growth of the gas phase *in vivo* following administration. Such contrast agent preparations may be

- 2 -

used to promote controllable and temporary retention of the gas phase, for example in the form of microbubbles, in tissue microvasculature, thereby enhancing the concentration of gas in such tissue and accordingly 5 enhancing its echogenicity, e.g. relative to the blood pool.

Such use of gas as a deposited perfusion tracer differs markedly from existing proposals regarding 10 intravenously administrable microbubble ultrasound contrast agents. Thus it is generally thought necessary to avoid microbubble growth since, if uncontrolled, this may lead to potentially hazardous tissue embolisation. Accordingly it may be necessary to limit the dose 15 administered and/or to use gas mixtures with compositions selected so as to minimise bubble growth *in vivo* by inhibiting inward diffusion of blood gases into the microbubbles (see e.g. WO-A-9503835 and WO-A-9516467).

In accordance with WO-A-9817324, on the other hand, 20 a composition comprising a dispersed gas phase is coadministered with a composition comprising at least one substance which has or is capable of generating a gas or vapour pressure *in vivo* sufficient to promote controllable growth of the said dispersed gas phase 25 through inward diffusion thereto of molecules of gas or vapour derived from said substance. This latter substance is referred to as a "diffusible component", although it will be appreciated that transport mechanisms other than diffusion may additionally or 30 alternatively be involved.

The preparations may advantageously be employed in visualising tissue perfusion in a subject, the increase in size of the dispersed gas being utilised to effect enrichment or temporary retention of gas in the 35 microvasculature of such tissue, thereby enhancing its echogenicity.

The dispersed gas content of such contrast agent

preparations will tend to be temporarily retained in tissue in concentrations proportional to the regional rate of tissue perfusion. Accordingly, when using 5 ultrasound imaging modalities such as conventional or harmonic B-mode imaging where the display is derived directly from return signal intensities, images of such tissue may be interpreted as perfusion maps in which the displayed signal intensity is a function of local 10 perfusion. This is in contrast to images obtained using free-flowing contrast agents, where the regional concentration of contrast agent and corresponding return signal intensity depend on the actual blood content 15 rather than the rate of perfusion of local tissue.

The present invention is based on the finding that 20 growth of a dispersed gas in order to bring about its temporary retention in tissue microvasculature may be effected by administering the gas dispersion in conjunction with one or more substances serving to destabilise the dispersion. By avoiding use of a 25 diffusible component requiring transport through blood and/or the continuous phase(s) in which the gas dispersion and diffusible component are contained, as well as through any encapsulating or other stabilising membranes which surround them, the invention may allow greater control of growth of the dispersed gas and may 30 also involve a higher proportion of gas-containing structures in the growth process.

Thus according to one aspect of the present 35 invention there is provided a combined preparation for simultaneous, separate or sequential use as a contrast agent in ultrasound imaging, said preparation comprising:

- i) an injectable aqueous gas dispersion, and
- ii) an administrable substance or substances 35 capable of destabilising said dispersed gas so as at least transiently to increase the size thereof.

According to a further aspect of the invention

there is provided a method of generating enhanced images of a human or non-human animal subject which comprises the steps of:

5       i) injecting a physiologically acceptable aqueous medium having gas dispersed therein into the vascular system of said subject;

10      ii) before, during or after injection of said aqueous medium administering to said subject a substance or substances capable of destabilising said dispersed gas so as at least transiently to increase the size thereof; and

15      iii) generating an ultrasound image of at least a part of said subject.

In general any biocompatible gas may be present in the gas dispersion used in accordance with the invention, the term "gas" as used herein including any substances (including mixtures) at least partially, e.g. substantially or completely, in gaseous or vapour form at the normal human body temperature of 37°C.

20      Representative gases thus include air; nitrogen; oxygen; carbon dioxide; hydrogen; inert gases such as helium, argon, xenon or krypton; sulphur fluorides such as sulphur hexafluoride, disulphur decafluoride or trifluoromethylsulphur pentafluoride; selenium

25      hexafluoride; optionally halogenated silanes such as methylsilane or dimethylsilane; low molecular weight hydrocarbons (e.g. containing up to 7 carbon atoms), for example alkanes such as methane, ethane, a propane, a butane or a pentane, cycloalkanes such as cyclopropane, cyclobutane or cyclopentane, alkenes such as ethylene, propene, propadiene or a butene, or alkynes such as acetylene or propyne; ethers such as dimethyl ether; ketones; esters; halogenated low molecular weight hydrocarbons (e.g. containing up to 7 carbon atoms); or mixtures of any of the foregoing. Advantageously at least some of the halogen atoms in halogenated gases are fluorine atoms; thus biocompatible halogenated

hydrocarbon gases may, for example, be selected from bromochlorodifluoromethane, chlorodifluoromethane, dichlorodifluoromethane, bromotrifluoromethane, chlorotrifluoromethane, chloropentafluoroethane, 5 dichlorotetrafluoroethane, chlorotrifluoroethylene, fluoroethylene, ethylfluoride, 1,1-difluoroethane and perfluorocarbons. Representative perfluorocarbons include perfluoroalkanes such as perfluoromethane, perfluoroethane, perfluoropropanes or perfluorobutanes 10 (e.g. perfluoro-n-butane, optionally in admixture with other isomers such as perfluoro-iso-butane); perfluoroalkenes such as perfluoropropene, perfluorobutenes (e.g. perfluorobut-2-ene), perfluorobutadiene, perfluoropentenes (e.g. 15 perfluoropent-1-ene) or perfluoro-4-methylpent-2-ene; perfluoroalkynes such as perfluorobut-2-yne; and perfluorocycloalkanes such as perfluorocyclobutane, perfluoromethylcyclobutane, perfluorodimethylcyclobutanes, perfluorotrimethyl- 20 cyclobutanes, perfluorocyclopentane, perfluoromethylcyclopentane, perfluorodimethylcyclopentanes, perfluorocyclohexane, perfluoromethylcyclohexane or perfluorocycloheptane. Other halogenated gases include methyl chloride, fluorinated (e.g. perfluorinated) 25 ketones such as perfluoroacetone and fluorinated (e.g. perfluorinated) ethers such as perfluorodiethyl ether. The use of perfluorinated gases, for example sulphur hexafluoride and perfluorocarbons such as perfluoropropane, perfluorobutanes, perfluoropentanes 30 and perfluorohexanes, may be particularly advantageous in view of the recognised high stability in the bloodstream of microbubbles containing such gases. Other gases with physicochemical characteristics which cause them to form highly stable microbubbles in the 35 bloodstream may likewise be useful.

The dispersed gas may, for example, be present in the form of microbubbles at least partially encapsulated

or otherwise stabilised by gas-stabilising material. This stabilising material may, for example, comprise an initially coalescence-resisting surface membrane (for example gelatin, e.g. as described in WO-A-8002365), a 5 filmogenic protein (for example an albumin such as human serum albumin, e.g. as described in US-A-4718433, US-A-4774958, US-A-4844882, EP-A-0359246, WO-A-9112823, WO-A-9205806, WO-A-9217213, WO-A-9406477 or WO-A-9501187), a polymer material (for example a synthetic biodegradable 10 polymer as described in EP-A-0398935, an elastic interfacial synthetic polymer membrane as described in EP-A-0458745, a microparticulate biodegradable polyaldehyde as described in EP-A-0441468, a microparticulate N-dicarboxylic acid derivative of a 15 polyamino acid - polycyclic imide as described in EP-A-0458079, or a biodegradable polymer as described in WO-A-9317718 or WO-A-9607434), a non-polymeric and non-polymerisable wall-forming material (for example as described in WO-A-9521631), or a surfactant (for example 20 a polyoxyethylene-polyoxypropylene block copolymer surfactant such as a Pluronic, a polymer surfactant as described in WO-A-9506518, or a film-forming surfactant such as a phospholipid, e.g. as described in WO-A-9211873, WO-A-9217212, WO-A-9222247, WO-A-9428780, WO-A-25 9503835 or WO-A-9729783).

Where phospholipid-containing gas dispersions are employed in accordance with the invention, e.g. in the form of phospholipid-stabilised gas microbubbles, representative examples of useful phospholipids include 30 lecithins (i.e. phosphatidylcholines), for example natural lecithins such as egg yolk lecithin or soya bean lecithin, semisynthetic (e.g. partially or fully hydrogenated) lecithins and synthetic lecithins such as dimyristoylphosphatidylcholine, 35 dipalmitoylphosphatidylcholine or distearoylphosphatidylcholine; phosphatidic acids; phosphatidylethanolamines; phosphatidylserines;

phosphatidylglycerols; phosphatidylinositols; cardiolipins; sphingomyelins; fluorinated analogues of any of the foregoing; mixtures of any of the foregoing and mixtures with other lipids such as cholesterol. The 5 use of phospholipids predominantly (e.g. at least 75%) comprising molecules individually bearing net overall charge, for example as described in WO-A-9729783, may be particularly advantageous. Representative negatively charged phospholipids include naturally occurring (e.g. 10 soya bean or egg yolk derived), semisynthetic (e.g. partially or fully hydrogenated) and synthetic phosphatidylserines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids and/or cardiolipins. Representative positively charged 15 phospholipids include esters of phosphatidic acids such as dipalmitoylphosphatidic acid or distearoyl-phosphatidic acid with aminoalcohols such as hydroxy-ethylenediamine.

The disclosures of all of the above-described 20 documents relating to gas-containing contrast agent formulations are incorporated herein by reference.

Gas microbubbles preferably have an initial average size not exceeding 10  $\mu\text{m}$  (e.g. of 7  $\mu\text{m}$  or less) in order to permit their free passage through the pulmonary 25 system following administration, e.g. by intravenous injection. However, larger microbubbles may be employed where, for example, these contain a mixture of one or more relatively blood-soluble or otherwise diffusible gases such as air, oxygen, nitrogen or carbon dioxide 30 with one or more substantially insoluble and non-diffusible gases such as perfluorocarbons. Outward diffusion of the soluble/diffusible gas content following administration will cause such microbubbles rapidly to shrink to a size which will be determined by 35 the amount of insoluble/non-diffusible gas present and which may be selected to permit passage of the resulting microbubbles through the lung capillaries of the

pulmonary system.

The destabilising substance(s) (hereinafter referred to as "the destabiliser") employed to induce at least transient growth of the dispersed gas phase may, 5 for example, operate by mechanisms involving (i) flocculation, aggregation or agglomeration whereby dispersed gas microbubbles assemble together to form clusters; (ii) coalescence or fusion of the dispersed gas moieties whereby two or more such moieties merge to 10 create a larger moiety; or (iii) Ostwald ripening of dispersed gas moieties, where larger microbubbles grow at the expense of smaller ones, the driving force being the higher pressure in small microbubbles compared to 15 large microbubbles as a result of differences in surface tension in accordance with Laplace's law. It will be appreciated that two or more mechanisms may operate simultaneously.

Flocculation, aggregation or agglomeration may be achieved by making the interbubble repulsive forces 20 weaker than the interbubble attractive forces. The size of the resulting clusters may depend on factors such as the initial size and concentration of the microbubbles, the hydrodynamic behaviour of the surrounding blood stream, the ambient temperature and the net attractive 25 force between the microbubbles.

Where the dispersed gas is stabilised by charge interactions, for example by interactions between charged surface groups in stabilising membranes of 30 materials such as charged phospholipids, addition of charged species such as electrolytes will supply counter-ions which will screen the charged surface groups and reduce interbubble repulsive forces. The use of di- and multi-valent counter-ions is preferred; thus, for example, salts, e.g. inorganic salts such as calcium 35 chloride or magnesium chloride may be used to destabilise negatively charged membranes.

Aggregation may also be induced by modifying the

properties of the surrounding water phase, for example by addition of solvents or of substances which change the electrical permeability of the medium so as to destabilise charge-stabilised gas dispersions.

5 Representative examples of such substances include water-miscible liquids or solids, e.g. aliphatic alcohols such as ethanol, isopropanol, ethylene glycol, propylene glycol, glycerol and sorbitol; aliphatic aldehydes and ketones such as acetaldehyde and acetone; 10 aliphatic esters such as methyl acetate, propyl formate and ethyl acetate; aliphatic ethers such as methyl propyl ether and di-isopropyl ether; aliphatic amides such as N,N-dimethylformamide and N,N-dimethylacetamide; aliphatic nitriles such as acetonitrile; carbohydrates 15 such as glucose and sucrose; polyethylene glycol; polypropylene glycol etc. Aliphatic compounds as described herein typically contain up to 6 carbon atoms.

Other representative destabilisers include components capable of bridging dispersed gas 20 microbubbles. Examples of such components include polymers, e.g. polysaccharides such as dextran and starches; polyaminoacids such as polylysine and proteins such as gelatin and albumin; polyethers such as polyethylene glycol, polypropylene glycol and 25 polyoxyethylene-polyoxypropylene block copolymers such as Pluronics; polyvinyl pyrrolidone etc.

Aggregation may also be induced by adding components which enter and modify the membrane, thereby altering the forces between different microbubbles. 30 Examples of such compounds include fatty alcohols, fatty acids and fatty amines; various surfactants; steroids such as cholesterol etc.

Where the gas-stabilising material contains acid or base groups on its surface, destabilisation may be 35 effected by addition of an appropriate acidic or basic destabiliser in order to change the pH.

Aggregation may be a reversible or irreversible

process, depending on the magnitude of the forces between the dispersed gas moieties. By optimising these forces the aggregates may be made loose enough to be easily disintegrated a short time after their temporary retention in tissue microvasculature, thus ensuring that the imaging procedure has a high safety profile. Such disintegration or redispersion of the aggregates may if desired be induced or enhanced by subsequent administration of an appropriate substance. For instance, a complexing agent such as ethylenediamine tetraacetic acid may remove or neutralise the effect of divalent or polyvalent ions used to destabilise charged microbubbles. A buffer, acid or base may be used to remove the effect of pH-induced aggregation/destabilisation. Solvents, e.g. alcohols such as ethanol, isopropanol, ethylene glycol, propylene glycol and glycerol; aldehydes and ketones such as acetaldehyde and acetone; ethers such as methyl propyl ether and di-isopropyl ether; aliphatic amides such as N,N-dimethylformamide and N,N-dimethylacetamide; or aliphatic nitriles such as acetonitrile, may for example be added to change the bridging behaviour of a polymeric flocculant by altering the polymer's ability to coil in the solvent. Such substances therefore re-establish the repulsive forces between the dispersed gas moieties.

Destabilisers which operate by inducing coalescence or fusion of the dispersed gas moieties include substances which modify the properties of gas-stabilising membrane material so that membrane rupture between coalescing gas moieties is enhanced. Representative destabilisers of this type include surfactants, co-surfactants, solvents and other substances which penetrate or otherwise modify the properties of the stabilising membrane. Chemical modification of the stabilising membrane, e.g. by addition of electrolytes or by pH change, or chemical reaction within the stabilising membrane may likewise be

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used to induce flocculation.

Growth of dispersed gas moieties by Ostwald ripening may be initiated by increasing the solubility of the gas in the surrounding aqueous phase, for example by adding a solvent or other appropriate component, e.g. a hydrotrope such as sodium benzoate or acetic acid, or a surfactant capable of solubilising the gas within micelles. Representative examples of solvents which may increase the solubility of the gas in the surrounding aqueous phase include aliphatic ethers such as ethyl methyl ether and methyl propyl ether; aliphatic esters such as methyl acetate, methyl formate and ethyl formate; aliphatic ketones such as acetone; aliphatic amides such as N,N-dimethylformamide and N,N-dimethylacetamide; and aliphatic nitriles such as acetonitrile. Representative examples of surfactants which may increase the solubility of the gas in the surrounding aqueous phase are those forming micelles in water, e.g. alkyl carboxylates, alkyl sulphonates, alkyl sulphates, dialkyl sulphosuccinates, alkylpyridinium salts, alkylammonium salts, alkyl polyethylene glycol ethers, alkyl polyethylene glycol esters and sorbitol fatty acid esters; alkyl groups in such surfactants may, for example, contain 5-30 carbon atoms.

Ostwald ripening kinetics may also be enhanced by increasing the interfacial tension about the dispersed gas, for example by modifying the properties of stabilising membranes, e.g. through solvent addition, salt addition, pH change or addition of surfactants/ co-surfactants which provide higher surface tension.

As noted above, the destabiliser may be administered before, during or after injection of the gas dispersion; the order of administration and timing between them when they are separate may be varied to permit control over the time, rate and thus location of temporary retention of the dispersed gas.

In many instances the destabiliser will be

administered by injection, e.g. intravenously, although intramuscular or subcutaneous injections may be useful in order to localise the effect of the destabiliser. 5 Volatile destabilisers, e.g. solvents such as ethanol, may, however, also be administered by inhalation; this may be of advantage in avoiding unwanted growth of the dispersed gas in the lung capillaries.

10 Imaging modalities which may be used in accordance with the invention include two- and three-dimensional imaging techniques such as B-mode imaging (for example using the time-varying amplitude of the signal envelope generated from the fundamental frequency of the emitted ultrasound pulse, from sub-harmonics or higher harmonics thereof or from sum or difference frequencies derived 15 from the emitted pulse and such harmonics, images generated from the fundamental frequency or the second harmonic thereof being preferred), colour Doppler imaging, Doppler amplitude imaging and combinations of these last two techniques with any of the other 20 modalities described above. To reduce the effects of movement, successive images of tissues such as the heart or kidney may be collected with the aid of suitable synchronisation techniques (e.g. gating to the ECG or respiratory movement of the subject). Measurement of 25 changes in resonance frequency or frequency absorption which accompany growth of the dispersed gas may also usefully be made to detect the contrast agent.

30 It will be appreciated that the dispersed gas content of combined contrast agent preparations according to the invention will, following 35 destabilisation, tend to be temporarily retained in tissue in concentrations proportional to the regional rate of tissue perfusion. Accordingly, when using ultrasound imaging modalities such as conventional or harmonic B-mode imaging where the display is derived directly from return signal intensities, images of such tissue may be interpreted as perfusion maps in which the

5 displayed signal intensity is a function of local perfusion. This is in contrast to images obtained using free-flowing contrast agents, where the regional concentration of contrast agent and corresponding return signal intensity depend on the actual blood content rather than the rate of perfusion of local tissue.

10 In cardiac studies, where perfusion maps are derived from return signal intensities in accordance with this embodiment of the invention, it may be advantageous to subject a patient to physical or pharmacological stress in order to enhance the distinction, and thus the difference in image 15 intensities, between normally perfused myocardium and any myocardial regions supplied by stenotic arteries.

15 As is known from radionuclide cardiac imaging, such stress induces vasodilatation and increased blood flow in healthy myocardial tissue, whereas blood flow in underperfused tissue supplied by a stenotic artery is substantially unchanged since the capacity for 20 arteriolar vasodilatation is already exhausted by inherent autoregulation seeking to increase the restricted blood flow.

25 The application of stress as physical exercise or pharmacologically by administration of adrenergic agonists may cause discomfort such as chest pains in patient groups potentially suffering from heart disease, and it is therefore preferable to enhance the perfusion 30 of healthy tissue by administration of a vasodilating drug, for example selected from adenosine, dipyridamole, nitroglycerine, isosorbide mononitrate, prazosin, doxazosin, dihydralazine, hydralazine, sodium nitroprusside, pentoxyphylline, amelodipine, felodipine, isradipine, nifedipine, nimodipine, verapamil, diltiazem and nitrous oxide. In the case of adenosine this may 35 lead to in excess of fourfold increases in coronary blood flow in healthy myocardial tissue, greatly increasing the uptake and temporary retention of

contrast agents in accordance with the invention and thus significantly increasing the difference in return signal intensities between normal and hypoperfused myocardial tissue. Because an essentially physical entrapment process is involved, retention of contrast agents according to the invention is highly efficient; this may be compared to the uptake of radionuclide tracers such as thallium 201 and technetium sestamibi, which is limited by low contact time between tracer and tissue and so may require maintenance of vasodilatation for the whole period of blood pool distribution for the tracer (e.g. 4-6 minutes for thallium scintigraphy) to ensure optimum effect. The contrast agents of the invention, on the other hand, do not suffer such diffusion or transport limitations, and since their retention in myocardial tissue may also rapidly be terminated by the methods described above, the period of vasodilatation needed to achieve cardiac perfusion imaging in accordance with this embodiment of the invention may be very short, for example less than one minute. This will reduce the duration of any possible discomfort caused to patients by administration of vasodilator drugs.

In view of the fact that the required vasodilatation need only be short lasting, adenosine is a particularly useful vasodilating drug, being both an endogenous substance and having a very short-lasting action as evidenced by a blood pool half-life of only 2 seconds. Vasodilatation will accordingly be most intense in the heart, since the drug will tend to reach more distal tissues in less than pharmacologically active concentrations. It will be appreciated that because of this short half-life, repeated injection or infusion of adenosine may be necessary during cardiac imaging in accordance with this embodiment of the invention; by way of example, an initial administration of 150 µg/kg of adenosine may be made substantially

simultaneously with administration of the contrast agent composition, followed 10 seconds later by slow injection of a further 150 µg/kg of adenosine, e.g. over a period of 20 seconds.

5       Contrast agent preparations in accordance with the invention may advantageously be employed as delivery agents for bioactive moieties such as therapeutic drugs (i.e. agents having a beneficial effect on a specific disease in a living human or non-human animal),  
10 particularly to targeted sites. Thus, for example, therapeutic compounds may be present in the dispersed gas or may be linked to part of the stabilising material, e.g. through covalent or ionic bonds, if desired through a spacer arm.

15       The controllable growth properties of the dispersed gas may be utilised to bring about its temporary retention in the microvasculature of a target region of interest, and thus retention of the gas and associated therapeutic compound in a target structure is  
20 particularly advantageous.

25       The therapeutic compound, which may if desired be coupled to a site-specific vector having affinity for specific cells, structures or pathological sites, may be released as a result of, for example, stretching or fracture of the gas-stabilising material caused by growth of the dispersed gas, solubilisation of the stabilising material, or disintegration of gas-microbubbles or gas-containing microparticles. Where a therapeutic agent is chemically linked to the gas-stabilising material, the linkage or any spacer arm associated therewith may advantageously contain one or more labile groups which are cleavable to release the agent. Representative cleavable groups include amide, imide, imine, ester, anhydride, acetal, carbamate, carbonate, carbonate ester and disulphide groups which are biodegradable *in vivo*, e.g. as a result of hydrolytic and/or enzymatic action.

Representative and non-limiting examples of drugs useful in accordance with this embodiment of the invention include antineoplastic agents such as vincristine, vinblastine, vindesine, busulfan, 5 chlorambucil, spiroplatin, cisplatin, carboplatin, methotrexate, adriamycin, mitomycin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopurine, mitotane, procarbazine, dactinomycin (anticinomycin D), daunorubicin, doxorubicin hydrochloride, taxol, 10 plicamycin, aminoglutethimide, estramustine, flutamide, leuprolide, megestrol acetate, tamoxifen, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase), etoposide, interferon a-2a and 2b, blood products such as hematoporphyrins or derivatives of the 15 foregoing; biological response modifiers such as muramylpeptides; antifungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine, miconazole or amphotericin B; hormones or hormone analogues such as growth hormone, melanocyte stimulating 20 hormone, estradiol, beclomethasone dipropionate, betamethasone, cortisone acetate, dexamethasone, flunisolide, hydrocortisone, methylprednisolone, paramethasone acetate, prednisolone, prednisone, 25 triamcinolone or fludrocortisone acetate; vitamins such as cyanocobalamin or retinoids; enzymes such as alkaline phosphatase or manganese superoxide dismutase; antiallergic agents such as amelexanox; anticoagulation 30 agents such as warfarin, phenprocoumon or heparin; antithrombotic agents; circulatory drugs such as propranolol; metabolic potentiators such as glutathione; antituberculars such as p-aminosalicylic acid, 35 isoniazid, capreomycin sulfate, cycloserine, ethambutol, ethionamide, pyrazinamide, rifampin or streptomycin sulphate; antivirals such as acyclovir, amantadine, azidothymidine, ribavirin or vidarabine; blood vessel dilating agents such as diltiazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide

dinitrate, nitroglycerin or pentaerythritol tetranitrate; antibiotics such as dapsone, chloramphenicol, neomycin, cefaclor, cefadroxil, cephalexin, cephadrine, erythromycin, clindamycin, 5 lincomycin, amoxicillin, ampicillin, bacampicillin, carbenicillin, dicloxacillin, cyclacillin, picloxacillin, hetacillin, methicillin, nafcillin, penicillin or tetracycline; antiinflammatories such as diflunisal, ibuprofen, indomethacin, meclofenamate, 10 mefenamic acid, naproxen, phenylbutazone, piroxicam, tolmetin, aspirin or salicylates; antiprotozoans such as chloroquine, metronidazole, quinine or meglumine antimonate; antirheumatics such as penicillamine; narcotics such as paregoric; opiates such as codeine, 15 morphine or opium; cardiac glycosides such as deslaneside, digitoxin, digoxin, digitalin or digitalis; neuromuscular blockers such as atracurium mesylate, gallamine triethiodide, hexafluorenium bromide, metocurine iodide, pancuronium bromide, succinylcholine 20 chloride, tubocurarine chloride or vecuronium bromide; sedatives such as amobarbital, amobarbital sodium, apropbarbital, butabarbital sodium, chloral hydrate, ethchlorvynol, ethinamate, flurazepam hydrochloride, glutethimide, methotriimeprazine hydrochloride, 25 methyprylon, midazolam hydrochloride, paraldehyde, pentobarbital, secobarbital sodium, talbutal, temazepam or triazolam; local anaesthetics such as bupivacaine, chloroprocaine, etidocaine, lidocaine, mepivacaine, procaine or tetracaine; general anaesthetics such as 30 droperidol, etomidate, fentanyl citrate with droperidol, ketamine hydrochloride, methohexitol sodium or thiopental and pharmaceutically acceptable salts (e.g. acid addition salts such as the hydrochloride or hydrobromide or base salts such as sodium, calcium or 35 magnesium salts) or derivatives (e.g. acetates) thereof; and radiochemicals, e.g. comprising beta-emitters. Of particular importance are antithrombotic agents such as

vitamin K antagonists, heparin and agents with heparin-like activity such as antithrombin III, dalteparin and enoxaparin; blood platelet aggregation inhibitors such as ticlopidine, aspirin, dipyridamole, iloprost and 5 abciximab; and thrombolytic enzymes such as streptokinase and plasminogen activator. Other examples of therapeutics include genetic material such as nucleic acids, RNA, and DNA of natural or synthetic origin, including recombinant RNA and DNA. DNA encoding certain 10 proteins may be used in the treatment of many different types of diseases. For example, tumour necrosis factor or interleukin-2 may be provided to treat advanced cancers; thymidine kinase may be provided to treat ovarian cancer or brain tumors; interleukin-2 may be 15 provided to treat neuroblastoma, malignant melanoma or kidney cancer; and interleukin-4 may be provided to treat cancer.

Contrast agent preparations in accordance with the 20 invention may be used as vehicles for contrast-enhancing moieties for imaging modalities other than ultrasound, for example X-ray, light imaging, magnetic resonance and, more preferably, scintigraphic imaging agents. Controlled growth of the dispersed gas phase may be used 25 to position such agents in areas of interest within the bodies of subjects, which may then be imaged.

Contrast agent preparations in accordance with the 30 invention may also be used as vehicles for therapeutically active substances which do not necessarily require release from the preparation in order to exhibit their therapeutic effect. Such preparations may, for example, incorporate radioactive atoms or ions such as beta-emitters which exhibit a localised radiation-emitting effect following growth of the dispersed gas phase and temporary retention of the 35 agent at a target site. It will be appreciated that such agents should preferably be designed so that subsequent shrinkage and cessation of retention of the

dispersed gas does not occur until the desired therapeutic radiation dosage has been administered.

Contrast agent preparations in accordance with the invention may additionally exhibit therapeutic properties in their own right. Thus, for example, the dispersed gas may be targeted to capillaries leading to tumours and may act as cell toxic agents by blocking such capillaries. Concentrations of dispersed gas in capillaries may also enhance absorption of ultrasonic energy in hyperthermic therapy; this may be used in, for example, treatment of liver tumours. Irradiation with a relatively high energy (e.g. 5 W) focused ultrasound beam, e.g. at 1.5 MHz, may be appropriate in such applications.

The following non-limitative Examples serve to illustrate the invention.

Preparation 1Phosphatidylserine-stabilised perfluorobutane  
microbubble dispersion

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Hydrogenated phosphatidylserine (5 mg/ml in a 1% w/w solution of propylene glycol in purified water) and perfluorobutane gas were homogenised in-line at 7800 rpm and ca. 40°C to yield a creamy-white microbubble dispersion. The dispersion was fractionated to substantially remove undersized microbubbles (<2 µm) and the volume of the dispersion was adjusted to the desired microbubble concentration by adding aqueous sucrose to give a sucrose concentration of 92 mg/ml. 2 ml portions of the resulting dispersion were filled into 10 ml flat-bottomed vials specially designed for lyophilisation, and the contents were lyophilised to give a white porous cake. The lyophilisation chamber was then filled with perfluorobutane and the vials were sealed. Prior to use, water was added to a vial and the contents were gently hand-shaken for several seconds to give a perfluorobutane microbubble dispersion.

25

Preparation 2Phosphatidylserine-stabilised perfluorobutane  
microbubble dispersion

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Hydrogenated egg phosphatidylserine (50 mg) was dispersed in water (9.75 ml, reversed osmosis quality) containing propylene glycol (0.25 ml) by heating to 80°C for about 2 minutes, followed by cooling to room temperature. 1 ml portions of the dispersion were transferred into ten 2 ml chromatography vials. The headspaces were flushed with perfluorobutane gas and the vials were capped and shaken on an Espe CapMix® shaker for 45 seconds, yielding samples of a milky white

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microbubble dispersion. The microbubble dispersions were collected in two 7 ml vials which were capped and placed on a roller table for two days. Later the samples were stored standing at room temperature until 5 use.

Preparation 3

Sodium dodecyl sulphate solution

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Sodium dodecyl sulphate (2.09 g) was added to water (7 ml, reversed osmosis quality) at room temperature to obtain a clear solution.

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Preparation 4

Brij 99 solution

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Brij 99 (2.84 g) was added to water (9.5 ml, reversed osmosis quality) and placed on a roller table overnight. A clear, slightly viscous solution was obtained.

Preparation 5

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Sodium dodecyl sulphate/n-butanol/water microemulsion

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Sodium dodecyl sulphate (12.5810 g), n-butanol (27.0813 g) and water (17.07 g, reversed osmosis quality) were mixed and diluted further with water to a water content of 30% w/w, yielding a clear, transparent microemulsion.

EXAMPLE 1

Microbubble aggregation by calcium chloride

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One drop of the microbubble dispersion prepared in Preparation 1 was placed on an object glass for

- 22 -

microscopy investigation. The sample was covered with a cover glass and placed under a microscope. Droplets of a 50 mg/ml calcium chloride solution in water were added to the edge of the cover glass so that the solution 5 penetrated into the microbubble dispersion. The behaviour of the microbubble dispersion as the calcium chloride solution front moved was recorded on a video tape. Microbubble aggregates with larger dimensions than the initial microbubbles were observed to form, 10 demonstrating that microbubbles with potential for retention in capillary systems were generated.

EXAMPLE 2

15 Microbubble aggregation by sodium dodecyl sulphate

One drop of the microbubble dispersion prepared in Preparation 2 was placed on an object glass and covered by a cover glass. The sample was placed under a Zeiss 20 Axioskop optical microscope equipped with a 10 x objective and a camera connected to a computer and an image analysis system. A droplet of the sodium dodecyl sulphate solution from Preparation 3 was placed on the edge of the coverglass, so that the solution penetrated 25 under the coverglass and into the microbubble dispersion. The behaviour of the microbubble dispersion as the sodium dodecyl sulphate solution front moved was recorded, showing formation of microbubble aggregates with larger dimensions than the initial microbubbles, 30 demonstrating the formation of microbubbles with potential for retention in capillary systems.

EXAMPLE 3

35 Microbubble aggregation by Brij 99

The procedure described in Example 2 was repeated using

the same microbubble dispersion from Preparation 2 but adding the Brij 99 surfactant solution from Preparation 4 to the edge of the coverglass. The experiment showed formation of microbubble aggregates with larger 5 dimensions than the initial microbubbles, demonstrating the formation of microbubbles with potential for retention in capillary systems.

EXAMPLE 4

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Microbubble growth by ethanol

The procedure described in Example 2 was repeated using 15 the same microbubble dispersions from Preparation 2 but adding pure ethanol to the edge of the coverglass. The experiment showed growth of microbubbles resulting in microbubbles of larger size than the initial microbubbles, thereby demonstrating the formation of 20 microbubbles with potential for retention in capillary systems.

EXAMPLE 5

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The procedure described in Example 2 was repeated using 30 the same microbubble dispersion from Preparation 2 but adding pure isopropanol to the edge of the coverglass. The experiment showed growth of microbubbles resulting in microbubbles of larger size than the initial microbubbles, thereby demonstrating the formation of microbubbles with potential for retention in capillary systems.

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EXAMPLE 6Microbubble growth by acetone

5 The procedure described in Example 2 was repeated using the same microbubble dispersion from Preparation 2 but adding pure acetone to the edge of the coverglass. The experiment showed growth of microbubbles resulting in microbubbles of larger size than the initial  
10 microbubbles, thereby demonstrating the formation of microbubbles with potential for retention in capillary systems.

EXAMPLE 7

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Microbubble aggregation and growth by a sodium dodecyl sulphate/n-butanol/water microemulsion

20 One droplet of the microbubble dispersion from Preparation 2, diluted three times with water was placed on an object glass and covered by a coverglass. The sample was placed under a Zeiss Axioskop optical microscope equipped with a 40 x objective and a camera connected to a computer and an image analysis system. A  
25 droplet of the sodium dodecyl sulphate/n-butanol/water microemulsion from Preparation 5 was placed on the edge of the coverglass, so that the microemulsion penetrated under the coverglass and into the microbubble dispersion. The behaviour of the microbubble dispersion as the microemulsion front advanced was recorded, showing formation of microbubble aggregates with larger dimensions than the initial microbubbles. A short time afterwards, the microbubbles in the aggregates fused or coalesced to give larger microbubbles, demonstrating the  
30 formation of microbubbles with potential for retention in capillary systems.  
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Claims:

1. A combined presentation for simultaneous, separate or sequential use as an ultrasound contrast agent, said preparation comprising:
  - i) an injectable aqueous gas dispersion; and
  - ii) an administrable substance or substances capable of destabilising said dispersed gas so as at least transiently to increase the size thereof.
2. A combined preparation as claimed in claim 1 wherein the dispersed gas comprises air, nitrogen, oxygen, carbon dioxide, hydrogen, an inert gas, a sulphur fluoride, selenium hexafluoride, an optionally halogenated silane, an optionally halogenated low molecular weight hydrocarbon, a ketone, an ester or a mixture of any of the foregoing.
3. A combined preparation as claimed in claim 2 wherein the dispersed gas comprises sulphur hexafluoride or a perfluorocarbon.
4. A combined preparation as claimed in claim 3 wherein said perfluorocarbon is perfluoropropane, perfluorobutane or perfluoropentane.
5. A combined preparation as claimed in any of the preceding claims wherein the dispersed gas is stabilised by an initially coalescence-resisting surface membrane, a filmogenic protein, a polymer material, a non-polymeric and non-polymerisable wall-forming material or a surfactant.
6. A combined preparation as claimed in claim 5 wherein said surfactant comprises at least one phospholipid.

7. A combined preparation as claimed in claim 6 wherein at least 75% of said surfactant comprises phospholipid molecules individually bearing net overall charge.

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8. A combined preparation as claimed in claim 7 wherein said charged phospholipid molecules are selected from phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, phosphatidic acid and cardiolipin molecules.

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10. A combined preparation as claimed in any of the preceding claims comprising one or more destabilising substances which induce growth of the dispersed gas by flocculation, aggregation, agglomeration, coalescence, fusion or Ostwald ripening.

11. A combined preparation as claimed in claim 10 comprising one or more destabilising substances selected from inorganic salts, aliphatic alcohols, aliphatic aldehydes, aliphatic ketones, aliphatic esters, aliphatic ethers, aliphatic amides, aliphatic nitriles, carbohydrates, polyethers, polysaccharides, polyaminoacids, polyvinylpyrrolidone, fatty alcohols, fatty acids, fatty amines, surfactants, steroids, acids, bases and hydrotropes.

12. A combined preparation as claimed in claim 11 comprising one or more destabilising substances selected from calcium chloride, magnesium chloride, ethanol, isopropanol, ethylene glycol, propylene glycol, glycerol, sorbitol, acetaldehyde, acetone, methyl formate, methyl acetate, propyl formate, ethyl acetate, ethyl methyl ether, methyl propyl ether, di-isopropyl ether, N,N-dimethylformamide, N,N-dimethylacetamide, acetonitrile, glucose, sucrose, polyethylene glycol, polypropylene glycol, polyoxyethylene-polyoxypropylene

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block copolymers, dextran, starches, polylysine, gelatin, cholesterol, and surface active alkyl carboxylates, alkyl sulphonates, alkyl sulphates, dialkyl sulphosuccinates, alkyl pyridinium salts, 5 alkylammonium salts, alkyl polyethylene glycol ethers, alkyl polyethylene glycol esters and sorbitol fatty acid esters.

10 13. A combined preparation as claimed in any of the preceding claims which further includes a vasodilator drug.

15 14. A combined preparation as claimed in claim 13 wherein said vasodilator drug is adenosine.

15 15. A combined preparation as claimed in any of claims 1 to 12 which further includes a therapeutic drug.

20 16. A combined preparation as claimed in any of claims 1 to 12 which further includes contrast-enhancing moieties for an imaging modality other than ultrasound.

25 17. A method of generating enhanced images of a human or non-human animal subject which comprises the steps of:

30 i) injecting a physiologically acceptable aqueous medium having gas dispersed therein into the vascular system of said subject;  
ii) before, during or after injection of said aqueous medium administering to said subject a substance or substances capable of destabilising said dispersed gas so as at least transiently to increase the size thereof; and  
35 iii) generating an ultrasound image of at least a part of said subject.

18. A method as claimed in claim 17 wherein

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destabilising substance is administered subcutaneously, intramuscularly, intravenously or by inhalation.

19. A method as claimed in claim 17 or claim 18 wherein  
5 a vasodilator drug is coadministered to the subject.

20. A method as claimed in claim 19 wherein said  
vasodilator drug is adenosine.

10 21. Use of a contrast agent as claimed in any of claims  
1 to 12 in ultrasound therapy.

15 22. Use as claimed in claim 21 wherein said therapy  
involves cell killing or blocking of blood flow to a  
site of interest.

19.05.00

Claims:

1. A combined presentation for simultaneous, separate or sequential use as an ultrasound contrast agent, said preparation comprising:
  - 5 i) an injectable aqueous gas dispersion; and
  - ii) a separately administrable substance or substances capable of destabilising said dispersed gas so as at least transiently to increase the size thereof.
- 10 2. A combined preparation as claimed in claim 1 wherein the dispersed gas comprises air, nitrogen, oxygen, carbon dioxide, hydrogen, an inert gas, a sulphur fluoride, selenium hexafluoride, an optionally halogenated silane, an optionally halogenated low molecular weight hydrocarbon, a ketone, an ester or a mixture of any of the foregoing.
- 15 20 3. A combined preparation as claimed in claim 2 wherein the dispersed gas comprises sulphur hexafluoride or a perfluorocarbon.
- 25 4. A combined preparation as claimed in claim 3 wherein said perfluorocarbon is perfluoropropane, perfluorobutane or perfluoropentane.
- 30 5. A combined preparation as claimed in any of the preceding claims wherein the dispersed gas is stabilised by an initially coalescence-resisting surface membrane, a filmogenic protein, a polymer material, a non-polymeric and non-polymerisable wall-forming material or a surfactant.
- 35 6. A combined preparation as claimed in claim 5 wherein said surfactant comprises at least one phospholipid.